Prevalence of Puroindoline Grain Hardness Genotypes among Historically Significant North American Spring and Winter Wheats

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ABSTRACT

Grain hardness ("hard" or "soft" kernel texture) is the single most important trait in determining the utilization and marketing of wheat (*Triticum aestivum* L.). Puroindoline a and b proteins represent the molecular basis for this trait. This study surveyed the prevalence of puroindoline hardness mutations (alleles) among North American spring and winter wheat varieties with emphasis on those that are historically important. Each variety was assessed for kernel texture using the Single Kernel Characterization System; *Hardness* alleles were defined by puroindoline gene sequence and the presence or absence of puroindoline a protein on polyacrylamide gels. A total of 90 spring wheats were examined: nine were soft and possessed wild-type ("soft") puroindoline sequences, 10 were mixed hardness, and the remaining 71 were uniformly hard. Of these hard spring wheats, 18 carried the *Pina-D1b* hardness allele (null for puroindoline a protein), 47 the *Pinb-D1b* allele (Gly-46–Ser-46), and four the *Pinb-D1c* allele (Leu-60–Pro-60). Two hard spring wheats possessed a new allele, designated *Pinb-D1e*, which involves a single nucleotide change in Trp-39 to a "stop" codon. Lastly, among the spring wheats, a new hardness allele was found in the hard component of the variety 'Utac' which was mixed. This allele, *Pinb-D1f*, also involved a single nucleotide change such that Trp-44 became a "stop" codon. A total of 62 winter wheat varieties were examined, of which five were soft and three were of mixed hardness. Of interest, the three mixed hardness wheats were 'Turkey', 'Kharkof', and 'Weston'. The hard component of each carried the *Pinb-D1b* allele. Of the 54 remaining wheats, all of which were hard, all but two carried this same *Pinb-D1b* allele. 'Chiefkan' winter wheat carried the same *Pinb-D1e* allele as 'Canadian Red' and 'Gehun' spring wheats. 'Andrews' hard red winter wheat possessed a new allele, designated *Pinb-D1g*, which was a single nucleotide change in Cys-56 to a "stop" codon. In conclusion, hard grain phenotype results from one of various mutations in either of the puroindoline proteins. To-date, seven hardness alleles have been discovered and characterized in hexapoid wheat. All but one occur in the puroindoline b gene coding sequence and result from single nucleotide changes. These molecular markers are useful in characterizing lineages and analyzing ancestral relationships.

Grain texture, that is whether the kernel is "hard" or "soft," is the primary means of classifying wheat for commerce since texture is the single most important trait in terms of end-use quality and utilization. Soft wheats are used for cakes, cookies, pastries, and some types of noodles, whereas hard wheats are used for breads and other yeast-leavened foods (Morris and Rose, 1996). The difference between hard and soft wheat kernel texture has been known to result from a single major gene on chromosome 5DS (Mattern et al., 1973; Law et al., 1978; Campbell et al., 1999).

The first insight into the molecular basis for kernel texture came with the discovery of friabilin. Friabilin, an M_r 15-kDa protein, is abundant on the surface of water-isolated soft wheat starch, scarce on the surface of hard wheat starch, and absent on durum (*Triticum durum* Desf.) (Greenwell and Schofield, 1986). Friabilin was found to be inherited additively according to Hardness allele dosage (abundant friabilin and soft kernel texture, *HaHaHa*, scarce friabilin and hard kernel texture, *hahaha*, and two intermediate levels of hardness and friabilin in heterozygous endosperm, *HaHaha* and *Hahaha*) (Bettge et al., 1995). The occurrence of friabilin was found to be mediated by bound polar lipids on the starch granule surface (Greenblatt et al., 1995). Morris et al. (1994) and Oda et al. (1992) later found that friabilin was actually more than one polypeptide. It is now well established that the main components of friabilin are, in fact, the Triton X-114 soluble proteins puroindolines a and b isolated by Blochet and co-workers (Blochet et al., 1991, 1993), and cloned and sequenced by Gautier et al. (1994) (Morris et al., 1994). The differential occurrence of friabilin on the surface of water-washed starch is apparently a partitioning phenomenon related to both the lipid binding properties of friabilin and the starch isolation procedure. Friabilin was shown to occur at roughly equivalent levels in both hard and soft wheat endosperm (Jolly et al., 1993). These apparently contradictory data were resolved with the discovery of two highly conserved hardness mutations in the puroindoline proteins (Giroux and Morris, 1997, 1998). The first puroindoline mutation that confers hardness is a null allele in puroindoline a, with no protein or mRNA transcript present (*Pina-D1b*) (Giroux and Morris, 1998) (Table 1). The second mutation is a single nucleotide base change in the codon of Gly–46, converting this amino acid to serine (*Pinb-D1b*) (Giroux and Morris, 1997). Two additional mutations in puroindoline b (*Pinb-D1c* and *Pinb-D1d*) were found during a survey of a large number of wheats of mostly Northern European origin (Lillemo and Morris, 2000).

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Abbreviations: aka, also known as; bp, base pair; CI, cereal introduction; CItr, cereal introduction, *Triticum*; Da, Dalton; GRIN, Germplasm Resources Information Network; PCR, polymerase chain reaction; PI, plant introduction; PNW, Pacific Northwest; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SKCS, Single Kernel Characterization System.

These two additional hardness mutations were both characterized as being single-nucleotide mutations and resulted in a change from Leu-60 to proline, and Trp-44 to arginine at these amino acid positions (Lillemo and Morris, 2000) (Table 1). Each puroindoline allele has been assigned a molecular marker designation (Table 1). The *Pina-D1b* allele was first characterized in the Australian cultivar Falcon and the *Pinb-D1b* allele was first characterized in the 'Chinese Spring' substitution line possessing the 5D chromosomes of 'Cheyenne' (Giroux and Morris, 1997, 1998). The Leu-60 to Pro-60 mutation was designated *Pinb-D1c*, and the second, Trp-44 to Arg-44, *Pinb-D1d*. The Arg-44 mutation was found in only three of 343 lines (Lillemo and Morris, 2000).

The two initial reports (Giroux and Morris, 1997, 1998) indicated that the two hardness mutations, *Pina-D1b* and *Pinb-D1b*, were highly conserved, and might explain most if not all phenotypically hard hexaploid wheats. Lillemo and Morris (2000) showed that the prevalence of these or other mutations might be largely related to the gene pools of interest and also the particular area of origin. In this report, we describe the prevalence of the four previously published puroindoline hardness alleles in a diverse set of North American hexaploid wheats of historical importance, with emphasis placed on including older, ancestral cultivars. Evidence of three new hardness alleles, all involving point mutations and causing null expression of puroindoline b, are presented.

MATERIALS AND METHODS

Seed Samples and Hardness Measurement

A subset of the "Pacific Northwest Historical Wheats Nursery" (Miller et al., 1990) developed by Dr. Ken Kephart was selected based on the "hard wheat" classification in that report (referenced in Tables 2 and 3; see also the "Commercial Wheat Cultivars" internet site at, gopher://greengenes.cit.cornell.edu:70/11/.cwc; verified August 9, 2000). Seed of these selected cultivars was harvested from plants grown under standard production practices at Spillman Farm, Washington State Univ., Pullman, WA, in 1990-1991 (winter wheats) and 1991 (spring wheats).

Additional cultivars of historical significance were selected on the basis of the report of Mercado et al. (1996) and Murphy et al. (1986) (Tables 2 and 3). For nearly all these cultivars, seed was obtained from Dr. Harold Bockelman of the USDA National Small Grains Collection (NSGC), Aberdeen, ID. The original 'Redman' CI 12496 was replaced with accession CI 012638. Additionally, the cultivars 'ID377s' (Table 2) and 'Bridger' (Table 3) were added. Breeder seed of ID377s was supplied by Dr. Ed Souza; seed of Bridger was obtained from the NSGC.

'Utac' was also grown in 1999 on experimental plots ("Barmore Research Farm") maintained by the Western Wheat Quality Laboratory in Pullman, WA, from seed obtained from the PNW Historical Nursery. Individual spikes were threshed and seed was analyzed by the SKCS and nucleic acid methods described below.

Grain hardness (kernel texture) was determined on an approximately 300-kernel subsample by the Perten Model SKCS 4100 Single Kernel Characterization System (Perten Instruments North America Inc., Springfield, IL). Although the SKCS data are not directly amenable to testing the significance of differences among cultivars because of lack of true replication, the very high level of expression of the hardness gene as measured by the SKCS qualitatively separates the soft and hard classes.

Table 1. Puroindoline a and b grain hardness (*Hardness*) (kernel texture) alleles, kernel phenotype, and molecular change in the puroindoline protein.

Puroindoline a	Puroindoline b	Phenotype, molecular change
Pina-D1a	*Pinb-D1a*	Soft, wild-type
Pina-D1b	*Pinb-D1a*	Hard, puroindoline a null
Pina-D1a	*Pinb-D1b*	Hard, puroindoline b, Gly-46 to Ser-46
Pina-D1a	*Pinb-D1c*	Hard, puroindoline b, Leu-60 to Pro-60
Pina-D1a	*Pinb-D1d*	Hard, puroindoline b, Trp-44 to Arg-44
Pina-D1a	*Pinb-D1e*	Hard, puroindoline b null, Trp-39 to stop codon
Pina-D1a	*Pinb-D1f*	Hard, puroindoline b null, Trp-44 to stop codon
Pina-D1a	*Pinb-D1g*	Hard, puroindoline b null, Cys-56 to stop codon

Protein Extraction and SDS-PAGE Detection of Puroindoline a Nulls

Triton-soluble proteins including puroindoline a and b were extracted from one to three crushed kernels with Triton X-114 detergent, separated by SDS-PAGE, and visually examined for the presence of puroindoline bands as described by Morris et al. (1994) and Giroux and Morris (1998).

DNA Isolation and Nucleic Acid-Based Detection of Puroindoline Alleles

Genomic DNA was isolated from each cultivar by the method of Dellaporta et al. (1983). Two tissues were used: leaf tissue from 10 to 15 plants each at the two- to three-leaf stage or individual half kernels (embryo half). The *Pinb-D1a* vs. *Pinb-D1b* allele alternatives (wild-type Gly-46 vs. the Ser-46 mutation) were assessed by means of sequence-specific primers which amplify allele-specific 250-bp fragments (Giroux and Morris, 1997). To assess the *Pinb-D1c* allele (Leu-60 to Pro-60), full length puroindoline b PCR product was restricted with *Pvu*II after the method of Lillemo and Morris (2000). All PCR products were visualized on 1.5% (w/v) agarose gels. Full-length puroindoline a and b were amplified with the primers described by Gautier et al. (1994), isolated from agarose gels after electrophoresis and sequenced by means of the amplification primers.

RESULTS

Spring Wheats

Nine spring wheat cultivars were classified as soft (hardness Class 5 or 4) by the SKCS on the basis of the four-class histogram Single Kernel Characterization System hardness data (SKCS hardness class, Table 2). The eight class "5" cultivars had mean and standard deviation hardness values consistent with soft wheat classification (means from 15–28, sd 12–18). 'Gypsum' was classed as "4" with a SKCS hardness score standard deviation of 20, typical of more highly variable seed lots; 14% of the kernels were ≥47 hardness. All these

Table 2. Historical spring wheat varieties arranged by SKCS hardness class, puroindoline genotype, and year of release.

Variety	PI/CI number	Ref.†	Year released or collected	Origin	Seed source‡	SKCS hardness Class	Mean ±sd	Frequency distribution (%) ≤33	34-46	47-59	≥60	Puroindoline allele§ *Pina-D1*	*Pinb-D1*
Soft Wheats (Class 5 or 4)													
Java	CI004966	2,7	1837	Russia	G	5	15 ± 18	85	11	2	2	A+	G+ S−
Steinwedel	CI004735	2	1890	Australia	G	5	24 ± 16	69	25	6	0	A+	G+ S−
Kota	PI192444	2,7	1903	Former Soviet U.	G	5	25 ± 15	65	28	7	0	A+	G+ S−
Aka Komugi	PI45234	2	1917	Japan	G	5	28 ± 16	60	30	7	3	A+	G+ S−
Surpresa	CI012474	2	1932	Brazil	G	5	27 ± 12	64	32	4	0	A+	G+ S−
Spinkcota	CI012375	1,4	1944	South Dakota	S	5	28 ± 14	64	28	7	1	A+	. S−
Pitic 62	CI013927	1,7	1962	Mexico	S	5	24 ± 15	77	19	3	1	A+	. S−
World Seeds 1	CI017347	1	1974	California	G	5	21 ± 17	76	16	7	1	A+	G+ S−
Gypsum	CI004762	2,7	1912	Colorado	G	4	29 ± 20	59	27	8	6	A+	G+ S−
Mixed Wheats (Class 3 or 2)													
Kenya	PI192171	2	1950	Kenya	G	3	51 ± 16	15	26	31	28	.	G− S+
Kenya 324	PI283840	2	1962	Kenya	G	3	44 ± 16	23	37	25	15	A+	G+ S−
Adams	CI013722	1,7	1968	Oregon	S	3	59 ± 20	12	11	23	54	A+	G− S+
Wampum	CI017691	1	1978	Washington	S	3	57 ± 20	13	14	21	52	.	. S+
Ladoga	CI004795	1,2,6,7	1886	Russia	G	2	66 ± 23	9	14	16	61	A+	. S− P+
Utac	CI010045	1,4	1928	Utah	G	2	49 ± 13	10	27	44	19	A+	G+ S− P−
Henry	CI012265	1,4,7	1944	Wisconsin	S	2	58 ± 18	7	14	33	46	A+	. S+
Sawtell	CI017424	1,7	1977	Idaho	S	2	64 ± 19	7	9	19	65	A+	G− S+
Kodiak Dwarf	NSL190931	1	1984	Montana	S	2	68 ± 22	8	9	14	69	A+	G− S+
Bronze Chief	NSL195357	1	1985	Montana	S	2	61 ± 21	9	11	25	55	A+	G− S+
Hard Wheats (Class 1) with *Pina-D1b*/*Pinb-D1a* Genotype													
Sea Island	CI006551	1,7	<1890	U.S.	S	1	77 ± 23	4	6	15	75	A−	G+ S−
Komar	CI008004	1,4,7	1930	North Dakota	S	1	79 ± 17	0	3	12	85	A−	G+ S−
Red Egyptian	CI012345	2	<1944	Egypt	G	1	77 ± 15	0	3	7	90	A−	G+ S−
Marroqui 588	PI168700	2	1948	Morocco	G	1	77 ± 16	0	2	9	89	A−	G+ S−
Lee	CI012488	1,4,6,7	1951	Minnesota	S	1	64 ± 15	2	9	23	66	A−	G+ S−
Chinook	CI13220	1,5,6,7	1952	Alberta	S	1	63 ± 14	1	9	27	63	A−	G+ S− P−
Fortuna	CI013596	1,7	1966	North Dakota	S	1	62 ± 15	1	12	32	55	A−	G+ S− P−
Fremont	CI014056	1,7	1970	Utah	S	1	79 ± 17	1	2	06	91	A−	G+ S−
Bounty 208	CI015078	1,7	1971	Colorado	S	1	71 ± 13	0	1	17	82	A−	G+ S− P−
Prodax	CI017407	1	1972	Minnesota	S	1	75 ± 15	0	4	9	87	A−	G+ S− P−
Olaf	CI015930	1	1973	North Dakota	S	1	67 ± 15	1	7	21	71	A−	G+ S− P−
Bounty 309	CI017315	1,7	1974	Colorado	S	1	80 ± 17	0	2	9	89	A−	G+ S−
Protor	CI017409	1	1975	Minnesota	S	1	59 ± 17	4	17	30	49	A−	G+ S−
Yecora Rojo	CI017414	1	1975	California	S	1	68 ± 17	5	3	14	78	A−	G+ S−
Aim	PVP7900005	1	1979	Montana	S	1	83 ± 15	0	0	5	95	A−	G+ S−
Westbred 906R	PI483455	1	1980	Montana	S	1	62 ± 17	5	10	25	60	A−	G+ S−
Westbred 911	PI483456	1	1981	Montana	S	1	73 ± 18	2	4	13	81	A−	G+ S−
ID377s	PI591045	–	1997	Idaho	B	1	81 ± 14	0	0	10	90	A−	G+ S−
Hard Wheats (Class 1) with *Pina-D1a*/*Pinb-D1b* Genotype													
Red Fife	PI348919	6,7	1842	Poland	G	1	77 ± 14	4	2	10	84	A+	G− S+
Fife	PI283820	2	1842	Poland	G	1	68 ± 16	0	2	30	68	A+	G− S+
Preston	CI003328	1,6,7	1893	Ontario	S	1	71 ± 16	1	4	14	81	A+	G− S+
Marquis	CI003641	1,6,7	1907	Ontario	S	1	70 ± 15	1	4	16	79	A+	G− S+
Ceres	CI006900	1,4,6,7	1926	North Dakota	S	1	82 ± 17	1	2	5	92	A+	G− S+
Reliance	CI007370	1,6,7	1926	Oregon	S	1	70 ± 19	3	11	13	73	A+	G− S+
Hope	CI008178	1,4,7	1927	South Dakota	S	1	68 ± 16	1	7	20	72	A+	G− S+
Reward	CI008182	1,6,7	1928	Ontario	S	1	67 ± 17	2	8	19	71	A+	G− S+
Flomar	CI011707	1	1933	Washington	S	1	70 ± 16	2	6	14	78	A+	G− S+
Canus	CI011637	1,4,6	1934	Alberta	S	1	72 ± 17	1	4	14	81	A+	G− S+
Thatcher	CI010003	1,4,6,7	1934	Minnesota	S	1	76 ± 14	0	2	12	86	A+	G− S+
Premier	CI011940	1,4	1938	North Dakota	S	1	68 ± 17	2	7	19	72	A+	G− S+
Pilot	CI011428	1,4,7	1939	North Dakota	S	1	70 ± 16	1	5	18	76	A+	G− S+
Regent	CI011869	1,4,6	1939	Manitoba	S	1	66 ± 16	3	7	22	68	A+	G− S+
Rival	CI011708	1,4,7	1939	North Dakota	S	1	70 ± 20	5	6	17	72	A+	G− S+
Comet	CI011465	1,4,7	1940	Montana	S	1	71 ± 17	3	5	14	78	A+	G− S+
Mida	CI012008	1,4,7	1944	North Dakota	S	1	57 ± 16	5	18	31	46	A+	G− S+
Cadet	CI012053	1,4,7	1946	North Dakota	S	1	72 ± 15	0	2	14	84	A+	G− S+
Rescue	CI012435	1,4,6,7	1946	Saskatchewan	S	1	61 ± 16	2	16	27	55	A+	G− S+
Redman	CI012638	1,4,6,7	1947	Manitoba	S	1	60 ± 16	4	16	27	53	A+	G− S+
Saunders	CI012567	1,4,6,7	1947	Ontario	S	1	74 ± 17	2	5	10	83	A+	G− S+
Rushmore	CI012273	1,4,7	1949	South Dakota	S	1	68 ± 16	2	4	25	69	A+	G− S+
Ramona 50	CI012390	1,5,7	1951	California	S	1	72 ± 16	1	7	13	79	A+	G− S+
Selkirk	CI013100	1,4,6,7	1953	Manitoba	S	1	60 ± 15	4	13	33	50	A+	G− S+
Conley	CI013157	1,4,7	1955	North Dakota	S	1	67 ± 17	3	9	19	69	A+	G− S+
Centana	CI012974	1,4,7	1958	Montana	S	1	60 ± 16	5	15	27	53	A+	G− S+
Canthatch	CI013345	1,4,7	1959	Manitoba	S	1	71 ± 18	3	5	13	79	A+	G− S+ P−
Justin	CI013462	1,4,7	1962	North Dakota	S	1	59 ± 14	2	14	35	49	A+	G− S+
Chris	CI13751	1,7	1965	Minnesota	S	1	64 ± 16	2	10	27	61	A+	G− S+
Manitou	CI013775	1,7	1965	Manitoba	S	1	68 ± 14	1	5	21	73	A+	G− S+
Moran	CI013743	1,7	1967	Idaho	S	1	63 ± 16	2	11	31	56	A+	G− S+
Red River 68	CI014193	1	1968	California	S	1	64 ± 17	4	11	20	65	A+	G− S− P−
Neepawa	CI015073	1,7	1969	Manitoba	S	1	70 ± 15	1	4	16	79	A+	G− S+
Era	CI013986	1,7	1970	Minnesota	S	1	72 ± 18	2	5	12	81	A+	G− S+

Continued.

Table 2. Continued.

Variety	PI/CI number	Ref.†	Year released or collected	Origin	Seed source‡	SKCS hardness Class	Mean ±sd	Frequency distribution (%) ≤33	34-46	47-59	≥60	Puroindoline allele§ *Pina-D1*	*Pinb-D1*
Anza	**CI015284**	**1**	**1971**	**California**	**S**	**1**	**67 ± 19**	**5**	**8**	**20**	**67**	**A+**	**G− S+**
Peak	**CI014587**	**1,7**	**1971**	**Idaho**	**S**	**1**	**65 ± 14**	**2**	**8**	**20**	**70**	**A+**	**G− S+**
Bannock	**CI015318**	**1,7**	**1972**	**Idaho**	**S**	**1**	**70 ± 16**	**2**	**5**	**20**	**73**	**A+**	**G− S+**
Peak 72	**CI015319**	**1,7**	**1972**	**Idaho**	**S**	**1**	**69 ± 13**	**0**	**4**	**16**	**80**	**A+**	**G− S+**
Wared	**CI015926**	**1,7**	**1972**	**Washington**	**S**	**1**	**76 ± 18**	**1**	**6**	**13**	**80**	**A+**	**G− S+**
Norana	**CI015927**	**1,7**	**1973**	**Montana**	**S**	**1**	**71 ± 16**	**1**	**4**	**15**	**80**	**A+**	**G− S+**
Borah	**CI017267**	**1,7**	**1974**	**Idaho**	**S**	**1**	**70 ± 14**	**2**	**3**	**14**	**81**	**A+**	**G− S+**
Kitt	**PI518818**	**1,7**	**1975**	**Minnesota**	**S**	**1**	**68 ± 14**	**1**	**4**	**20**	**75**	**A+**	**G− S+**
Prospur	**CI017408**	**1**	**1975**	**Minnesota**	**S**	**1**	**64 ± 16**	**3**	**7**	**22**	**68**	**A+**	**G− S+**
Newana	**CI017430**	**1,7**	**1976**	**Montana**	**S**	**1**	**72 ± 13**	**0**	**3**	**10**	**87**	**A+**	**G− S+**
Pondera	**CI017828**	**1,7**	**1980**	**Montana**	**S**	**1**	**70 ± 15**	**2**	**4**	**13**	**81**	**A+**	**G− S+**
Probrand 751	**PI486144**	**1**	**1980**	**Minnesota**	**S**	**1**	**63 ± 15**	**2**	**9**	**26**	**63**	**A+**	**G− S+**
McKay	**CI017903**	**1**	**1981**	**Idaho**	**G**	**1**	**82 ± 17**	**1**	**1**	**7**	**91**	**A+**	**G− S+**
Hard Wheats (Class 1) with *Pina-D1a*/*Pinb-D1c* Genotype													
Hard Red Calcutta	**CI015090**	**2,6**	**~1886**	**India**	**G**	**1**	**72 ± 17**	**2**	**6**	**12**	**80**	**A+**	**G+ S− P+**
Ruby	**CI006047**	**1,6,7**	**1917**	**Ontario**	**S**	**1**	**72 ± 14**	**3**	**6**	**21**	**70**	**A+**	**G+ S− P+**
Red Bobs	**CI006255**	**1,4,7**	**1918**	**Saskatchewan**	**S**	**1**	**77 ± 15**	**1**	**3**	**6**	**90**	**A+**	**G+ S− P+**
Supreme	**CI008026**	**1,4,7**	**1922**	**Saskatchewan**	**S**	**1**	**77 ± 16**	**1**	**2**	**9**	**88**	**A+**	**G+ S− P+**
Hard Wheats (Class 1) identified as puroindoline B nulls													
Gehun	**PI116066**	**2**	**<1891**	**India**	**G**	**1**	**67 ± 18**	**3**	**11**	**15**	**71**	**A+**	**G+ S−**
Canadian Red	**CI006282**	**1,7**	**1919**	**California**	**S**	**1**	**71 ± 15**	**1**	**5**	**11**	**83**	**A+**	**G+ S−**

† Reference to source and description, where 1 is Miller et al., 1990; 2 is Mercado et al., 1996; 3 is Murphy et al., 1986; 4 is Bayles and Clark, 1954; 5 is Briggle and Reitz, 1963; 6 is Fraser and Whiteside 1956; and 7 is CSSA, 1982.

‡ Seed source where "S" is PNW Historical Wheats Nursery, "G" is NSGC, and "B" is breeder seed.

§ Puroindoline alleles, where A+ and A− indicate presence of *Pina-D1a* and *Pina-D1b* alleles, respectively, based on SDS-PAGE detection of puroindoline a protein; G+ and G− indicate presence of *Pinb-D1a* and absence of *Pinb-D1a*, respectively, based on presence or absence of a 250-bp PCR product using Gly-46 specific primers; S+ and S− indicate presence of *Pinb-D1b* and absence of *Pinb-D1b*, respectively, based on presence or absence of a 250-bp PCR product using Ser-46 specific primers; P+ indicates presence of *Pinb-D1c* based on the cleavage of puroindoline b PCR product with *Pvu*II; P− indicates that the cleavage site associated with *Pinb-D1c* was not present; a "." indicates that no analysis was conducted. Puroindoline allele designations in some instances were determined by sequencing the puroindoline PCR-produced gene product.

soft wheats exhibited a puroindoline a band on SDS-PAGE (summarized in Table 2, gel data not shown). Of the seven randomly selected soft wheat cultivars checked by means of the "soft" wild-type Gly-46 specific PCR primer, all showed a product. None of the cultivars produced a PCR product with the "hard" Ser-46 specific primer (Table 2).

Table 2 indicates the reference source for these soft (Class 5 and 4) cultivars. All but 'Spinkcota', 'Pitic 62', and 'World Seeds 1' were described in Mercado et al. (1996). 'Aka Komugi' (PI 45234, also CI 6138) is listed as 'Akagomughi' by van Beuningen and Busch (1997), Mercado et al. (1996), and Borojević (1983). Spinkcota and Pitic 62 were listed by Miller et al. (1990) as hard red spring cultivars. Bayles and Clark (1954) described Spinkcota as having hard, red kernels. Seed from the NSGC exhibited the similar soft texture as observed with the seed from the PNW Historical Nursery (SKCS hardness = 37±17). The correct classification of this cultivar is unknown. Although Pitic 62 is also listed by Miller et al. (1990) as a hard red spring wheat, the presence of puroindoline a protein and absence of Ser-46 specific PCR product were all consistent with the SKCS soft texture phenotype. The original description of Pitic 62 (INIA and CIMMYT, 1972) describes the kernel as soft. World Seeds 1 was listed in the GRIN (http://www.ars-grin.gov/; verified August 9, 2000) and by Miller et al. (1990) as hard white spring, yet the SKCS data indicate that it is soft. Seed from both the PNW Historical Nursery and the NSGC were consistent in their soft character.

Ten spring wheat cultivars were classified as "mixed" (hardness Class 3 or 2, Table 2) by the SKCS. All Class 3 cultivars exhibited intermediate hardness values (44–59) and generally higher standard deviations (16–20). Six of the seven Class 2 cultivars had at least three-fourths of their kernels ≥47 and mean hardness values of 58-68.

Seed of Utac from the PNW Historical Nursery was classed as '3' with an SKCS hardness of 51±19 (data not shown). New seed obtained from NSGC was classed as '2' with an SKCS hardness of 49±13 (Table 2). Since none of these data provided a clear indication as to the homogeneity of this cultivar, new seed was propagated and individual spikes were analyzed for both hardness and puroindoline genotype. Individual spike data clearly revealed the hard and soft mixed nature of Utac. Of 8 spikes tested, 3 were classed as soft (Class 4 or 5) and 5 as hard (Class 1) (data not shown). Puroindoline a and b were sequenced from the hard component of Utac; the results revealed "wild-type" puroindoline a (*Pina-D1a*) but a previously unknown single-nucleotide mutation in puroindoline b (see Table 1). The point mutation causes Trp-44 to change to a stop codon (TGG to TGA). This allele was given the molecular marker designation, *Pinb-D1f*. Bayles and Clark (1954) described Utac as, "kernels white, semihard to hard" which would be consistent with a cultivar having a mixture of hard and soft kernels.

'Adams', 'Wampum', 'Henry', 'Sawtell', 'Kodiak Dwarf', and 'Bronze Chief' were grown as part of the Historical Wheat study (Miller et al., 1990). New seed was obtained from NSGC and gave similar, mixed hard and soft SKCS results (data not shown). In all six of these mixed cultivars the "hard" component exhibited

Table 3. Historical winter wheat varieties arranged by SKCS hardness class, puroindoline genotype, and year of release.

Variety	PI/CI number	Ref.†	Year released or collected	Origin	Seed source‡	SKCS hardness						Puroindoline alleles§		
						Class	Mean ±sd	Frequency distribution (%) ≤33	34-46	47-59	≥60	*Pina-D1*	*Pinb-D1*	
Soft Wheats (Class 5 or 4)														
Gold Drop	CI006316	1,7	1843	England	S	5	19 ± 18	80	14	3	3	A+	.	S−
PI178383	PI178383	1	<1949	Turkey	S	5	21 ± 17	77	17	5	1	A+	.	S−
Benhur	CI014054	3,7	1966	Indiana	G	5	25 ± 16	64	26	9	1	A+	G+	S−
Sherman	CI004430	1,4,7	1928	Oregon	S	4	37 ± 15	40	33	19	8	A+	.	S−
Lucas	CI012990	3,5,7	1959	Ohio	G	4	38 ± 18	41	26	23	10	A+	G+	S−
Mixed Wheats (Class 3 or 2)														
Turkey	CI001558	1,2,4,7	1873	Kansas (Russia)	G	3	47 ± 19	24	26	30	20	.	.	S+
Kharkof	CI001442	1,4	1900	Ukraine	G	3	30 ± 22	57	20	12	11	.	.	S+
Weston	CI017727	1	1978	Idaho	G	3	60 ± 18	11	7	27	55	.	.	S+
Hard Wheats (Class 1) with *Pina-D1a*/*Pinb-D1b* Genotype														
Montana No. 36	CI005549	1,4,7	1915	Montana	S	1	63 ± 15	2	10	30	58	A+	.	S+
Blackhull	CI006251	1,4,7	1917	Kansas	G	1	67 ± 15	3	6	13	78	.	.	S+
Kanred	CI005146	1,4,7	1917	Kansas	G	1	61 ± 16	5	8	31	56	.	.	S+
Ridit	CI006703	1,4,7	1923	Washington	S	1	61 ± 17	4	14	23	59	.	.	S+
Mosida	CI006688	1,4,7	1924	Idaho	G	1	71 ± 18	5	4	11	80	A+	G−	S+
Oro	CI008220	1,7	1927	Oregon	S	1	70 ± 15	1	4	20	75	.	G−	S+
Early Blackhull	CI008856	1,4,7	1928	Kansas	G	1	63 ± 16	3	8	33	56	.	.	S+
Rio	CI010061	1,4,7	1931	Oregon	S	1	68 ± 16	3	5	19	72	.	G−	S+
Tenmarq	CI006936	1,4,7	1932	Kansas	S	1	64 ± 16	1	13	25	61	.	.	S+
Yogo	CI008033	1,4,7	1932	Montana	G	1	74 ± 18	2	3	14	81	.	.	S+
Cheyenne	CI008885	1,4,7	1933	Nebraska	G	1	79 ± 14	0	2	3	95	.	.	S+
Relief	CI010082	1,4,7	1934	Utah	G	1	63 ± 16	2	15	22	61	.	.	S+
Cache	CI011599	1,4,7	1937	Utah	S	1	64 ± 16	4	9	21	66	.	.	S+
Triumph	CI012132	1,4,7	1940	Oklahoma	G	1	71 ± 12	0	1	15	84	.	.	S+
Pawnee	CI011669	1,4,7	1942	Nebraska	S	1	62 ± 15	1	11	30	58	.	G−	S+
Wasatch	CI011925	1,4	1942	Utah	S	1	62 ± 15	4	11	30	55	.	.	S+
Blue Jacket	CI012502	1,4	1946	Kansas	S	1	57 ± 15	5	14	38	43	.	G−	S+
Kiowa	CI012133	1,5,7	1950	Kansas	G	1	61 ± 13	1	11	28	60	.	.	S+
Columbia	CI012928	1,5,7	1955	Oregon	S	1	65 ± 15	2	6	28	64	.	.	S+
Bison	CI012518	1,5,7	1956	Kansas	S	1	59 ± 14	2	20	30	48	A+	G−	S+
Burt	CI012696	1,5,7	1956	Washington	S	1	65 ± 17	4	9	20	67	.	G−	S+
Itana	CI012933	1,5,7	1956	Montana	S	1	67 ± 16	3	9	20	68	.	G−	S+
Westmont	CI012930	1,5,7	1956	Montana	S	1	66 ± 19	4	8	22	66	.	.	S+
Tendoy	CI013426	1,5	1960	Idaho	S	1	68 ± 14	2	2	21	75	.	.	S+
Warrior	CI013190	3,7	1960	Nebraska	G	1	67 ± 18	2	5	25	68	.	G−	S+
Delmar	CI013442	1,5,7	1961	Utah	G	1	77 ± 16	0	2	12	86	.	.	S+
Scout	CI013546	3,7	1964	Nebraska	G	1	71 ± 16	3	4	17	76	.	G−	S+
Itana 65	CI013846	1	1965	Idaho	S	1	66 ± 15	1	9	26	64	.	.	S+
McCall	CI013842	1,7	1965	Washington	S	1	70 ± 19	3	9	12	76	.	.	S+
Wanser	CI013844	1,7	1965	Washington	S	1	69 ± 16	1	6	18	75	.	.	S+
Sturdy	CI013684	3,7	1966	Texas	G	1	64 ± 17	3	8	27	62	.	G−	S+
Crest	CI013880	1,7	1967	Montana	S	1	66 ± 20	5	9	16	70	.	G−	S+
Bridger	CI014580	7	1969	Utah	S	1	74 ± 16	2	3	10	85	.	G−	S+
Centurk	CI015075	3,7	1971	Nebraska	G	1	84 ± 13	0	0	02	98	A+	G−	S+
Coulee	CI014483	1,7	1971	Washington	G	1	74 ± 12	0	1	9	90	.	.	S+
Ark	CI015286	1	1972	Idaho	G	1	77 ± 16	1	4	9	86	A+	G−	S+
Franklin	CI015317	1,7	1972	Idaho	S	1	67 ± 15	2	9	22	67	A+	G−	S+
Ranger	CI015316	1,7	1972	Idaho	S	1	70 ± 17	0	6	22	72	.	G−	S+
Hansel	CI017296	1,7	1974	Utah	S	1	64 ± 16	5	6	22	67	.	.	S+
Heglar	CI017269	1,7	1974	Idaho	S	1	73 ± 17	0	8	8	84	.	G−	S+
Jeff	CI017270	1,7	1974	Idaho	S	1	66 ± 15	1	6	29	64	.	G−	S+
Arbon	CI017746	1,7	1978	Idaho	S	1	68 ± 16	2	5	18	75	A+	G−	S+
Hatton	CI017772	1	1979	Washington	S	1	73 ± 17	1	6	12	81	A+	G−	S+
Manning	CI017846	1,7	1979	Utah	S	1	69 ± 14	0	8	15	77	.	.	S+
Neeley	CI017860	1	1980	Idaho	S	1	69 ± 19	2	6	15	77	.	G−	S+
Winridge	CI017902	1	1981	Montana	S	1	68 ± 19	4	7	16	73	.	G−	S+
Ute	PI490017	1	1983	Utah	G	1	56 ± 14	3	23	40	34	.	.	S+
Norwin	PI491533	1	1984	Montana	S	1	65 ± 17	5	8	18	69	.	G−	S+
Batum	PI495013	1	1985	Washington	S	1	67 ± 20	5	10	20	65	.	G−	S+
Blizzard	PI512302	1	1989	Idaho	S	1	59 ± 14	2	14	36	48	.	G−	S+
Buchanan	PI532994	1	1989	Washington	S	1	69 ± 19	4	6	17	73	.	G−	S+
Survivor	PI509503	1	1991	Idaho	S	1	59 ± 14	3	13	34	50	.	G−	S+
Hard Wheats (Class 1) identified as puroindoline B nulls														
Chiefkan	CI011754	1,4	1935	Kansas	S	1	71 ± 15	1	3	14	82	A+	G+	S−
Andrews	PI512282	1	1987	Washington	G	1	70 ± 16	2	3	14	81	A+	G+	S−

† Reference to source and description, where 1 is Miller et al., 1990; 2 is Mercado et al., 1996; 3 is Murphy et al., 1986; 4 is Bayles and Clark, 1954; 5 is Briggle and Reitz, 1963, 6 is Fraser and Whiteside 1956; and 7 is CSSA, 1982.

‡ Seed source where "S" is PNW Historical Wheats Nursery, and "G" is NSGC.

§ Puroindoline alleles, where A+ indicates *Pina-D1a* allele based on SDS-PAGE detection of puroindoline a protein; G+ and G− indicate presence of *Pinb-D1a* and absence of *Pinb-D1a*, respectively, based on presence or absence of a 250-bp PCR product using Gly-46 specific primers; S+ and S− indicate presence of *Pinb-D1b* and absence of *Pinb-D1b*, respectively, based on presence or absence of a 250-bp PCR product using Ser-46 specific primers; a "." indicates that no analysis was conducted. Puroindoline allele designations in some instances were determined by sequencing the puroindoline PCR-produced gene product.

the prevalent Gly-46 to Ser-46 hardness mutation (Table 2). Adams is listed in its registration notice (Rohde, 1972) as a hard white spring derived from an F_4 bulk of the cross 'Idaed'/'Burt'. Idaed is a soft white spring and Burt a hard white winter, so it is possible that Adams was originally released as a hard and soft mixed (heterogeneous) cultivar. Adams and Burt share the *Pinb-D1b* hardness allele (Tables 2 and 3). Wampum was never registered. Henry is described by Bayles and Clark (1954) as "kernels semihard to hard" which is consistent with a hard and soft mixed composition. Sawtell has the pedigree 'Sonora 64'/'Winalta'. Sonora 64 and Winalta are listed on the Greengenes server (see Materials and Methods) as hard red spring and winter cultivars, respectively. The source of the soft component of Sawtell is unknown. Sawtell was developed by Don Sunderman at Aberdeen, ID. Kodiak Dwarf and Bronze Chief share the same pedigree ('McKay'/'Plainsman V'). New seed of Kodiak Dwarf from NSGC produced an SKCS Class 3 ("mixed") with a hardness value of 60±21. Greengenes lists Plainsman V as a hard red winter cultivar. The source of the soft component of Kodiak Dwarf and Bronze Chief are unknown; both cultivars originated at Great Plains Seed and Research, Inc., Bozeman, MT.

Original seed of McKay from the PNW Historical Nursery produced a "mixed" (Class 2) SKCS hardness value of 68±18 with about 15% soft kernels (<47). New seed of McKay from NSGC produced an SKCS hardness of 82±17 (Table 2).

The remaining three mixed class spring wheat cultivars, 'Kenya', 'Kenya 324' and 'Ladoga', were included due to their historical significance (Mercado et al., 1996). PCR results indicated that the "hard" component of Kenya possessed the Ser-46 *Pinb-D1b* allele (Table 2). A survey of four randomly-selected kernels of Kenya 324 produced only soft wild-type puroindoline b sequence. Regarding the "Kenya" cultivars it should be noted that over 10 accessions of "Kenya" exist in the NSGC.

The "hard" component of Ladoga exhibited puroindoline a protein on SDS-PAGE, produced no product using the Ser-46 specific PCR primer, but did exhibit the *Pvu*II restriction site characteristic of the Leu-60 to Pro-60 hardness allele (Table 2). Sequencing full-length puroindoline a and b PCR products confirmed this genotype (*Pina-D1a*, *Pinb-D1c*).

The majority (n = 71) of the spring wheat cultivars included in the study proved to be hard (Class 1, Table 2). SKCS hardness ranged from 57 for 'Mida' to 83 for 'Aim'. All but 'Red Egyptian', 'Marroqui 588', ID377s, 'Fife', 'Hard Red Calcutta', and 'Gehun' were included in the PNW Historical Wheat study and seed was obtained from Spillman Farm. Because 'Red Fife' was listed by Miller et al. (1990) as a soft red spring, it was not originally included in the PNW Historical Wheat set, but was obtained later from NSGC.

Of these 71 Class 1 hard spring wheat cultivars, 18 were shown to lack puroindoline a protein (Table 2) and therefore carry the *Pina-D1b* hardness mutation. PCR results conducted using both the Gly-46 and Ser-46 specific primers were consistent with this genotype, that is, all expressed the "soft" wild-type Gly-46 puroindoline b sequence. A check of five randomly-selected cultivars lacked the Leu-60 to Pro-60 hardness mutation.

The majority of the hard spring wheat cultivars (n = 47) produced the characteristic 250-bp PCR fragment using the Ser-46 specific primer. These results along with the presence of puroindoline a protein on SDS-PAGE and the lack of PCR product using the Gly-46 specific primer were all consistent with the presence of the *Pinb-D1b* hardness allele in these cultivars (Table 2). Initially, it was difficult to obtain clear PCR results with 'Canthatch' and 'Red River 68'. They were checked for the Leu-60 to Pro-60 mutation, found not to have it, and eventually had puroindoline a and b sequenced in their entirety. This sequencing confirmed their Ser-46 *Pinb-D1b* hardness genotype.

Of the remaining six Class 1 hard spring wheats, four (Hard Red Calcutta, 'Ruby', 'Red Bobs', and 'Supreme') exhibited the unique *Pvu*II cleavage site in puroindoline b associated with the Leu-60 to Pro-60 mutation (*Pinb-D1c* allele, Table 1) (Lillemo and Morris, 2000). These cultivars additionally showed puroindoline a protein (SDS-PAGE), the soft Gly-46 PCR product, and no Ser-46 specific PCR product (Table 2).

The last two hard spring cultivars, Gehun and 'Canadian Red', exhibited puroindoline a protein on SDS-PAGE, a Gly-46 specific PCR product, but no Ser-46 PCR product. New seed was obtained from NSGC and puroindoline a and b were sequenced. Both proved to have a single-nucleotide change in the codon of Trp-39 (TGG to TGA) which created a stop codon at this position. This hardness mutation was assigned the molecular marker designation, *Pinb-D1e* (Table 1).

Winter Wheats

Five winter wheats were shown to be soft by SKCS analysis (Class 5 or 4, Table 3); hardness values ranged from 19 to 38. All showed both puroindoline a and b proteins on SDS-PAGE, and no product using the Ser-46 specific PCR primer. As a control, 'Benhur' and 'Lucas' were checked using the Gly-46 specific primer and the characteristic 250-bp product was observed. Benhur and Lucas are described as soft red winter cultivars (Patterson et al., 1978; Heyne, 1960; respectively). 'Gold Drop', 'PI 178383', and 'Sherman' were listed by Miller et al. (1990) as hard red winter cultivars. Although Gold Drop is also listed as a hard red winter wheat on the Greengenes web site, Clark (1927) lists it as a soft red winter wheat, as does GRIN which also describes kernel color as "white/amber," but gives no other indication as to kernel texture. Bayles and Clark (1954) describe Sherman as having "semihard" kernels, but go on to state, "Sherman differs from Turkey chiefly in having.... softer kernels,..." Sherman is listed by Bayles and Clark (1954) as being derived from a cross involving 'Turkey', 'Budapest' and 'Zimmerman'. Budapest appears in the GRIN as PI 11227 (inactive status, received 1904 from the Agricultural Experiment Station, Manhattan, KS). Its kernel texture is unknown. Zimmerman appears in GRIN as NSL34203, origin Oregon. Bayles and Clark (1954) describe this same Zimmerman as also known

as 'Oregon Zimmerman', and cautioned that, "It... should not be confused with the soft red winter cultivar bearing the latter [Zimmerman] name." Since Sherman originated at the Sherman Experiment Station in Oregon, it more likely involved the Oregon Zimmerman. Irrespective of *which* Zimmerman was indeed used in the cross to produce Sherman, a soft allele could have been inherited and present in the cultivar.

Only three winter wheat cultivars were classed as "mixed" by the SKCS (Class 2 or 3, Table 3). Of interest, both Turkey (aka 'Turkey Red') and 'Kharkof' were in this class. New seed was obtained from the NSGC and produced similar results (data not shown). Turkey had approximately 50% kernels ≥ 47 hardness, whereas in Kharkof, this percentage of hard kernels was only 23%. New NSGC seed of 'Weston' was also classed as mixed. The hard and soft mixed nature of Turkey, Kharkof and Weston was unexpected. Bayles and Clark (1954) describe Turkey as having, "kernels red, midlong, hard,..." Their discussion of Kharkof does not indicate kernel texture. The GRIN lists Weston as being a hard red winter wheat derived from the cross 'Bezostaya'/2/Burt/PI 178383. 'Bezostaja' (PI 323468, the two spellings are considered equivalent) is listed in the GRIN as a hard red winter, Burt is a hard white winter (Heyne, 1959) and PI 178383 is a soft wheat (Table 3). Burt carries the *Pinb-D1b* allele (Table 3); Weston may have been released as a heterogeneous mixture of the soft allele from PI 178383 and the hard allele from Burt (or Bezostaja).

A significant number (ca. 17) of other winter wheat seed samples derived from the PNW Historical Nursery were characterized as being "mixed" (Class 2 or 3) (data not shown). These cultivars can be identified in Table 3 as having reference 1 (Miller et al., 1990), but having seed source listed as "G" (NSGC). In all cases, except the three cultivars just described as mixed, all new seed stocks from NSGC confirmed the uniformly hard (Class 1) phenotype (Table 3).

Nearly all (52 of 54) of the hard winter wheats (SKCS Class 1) (Table 3) produced a 250-bp product using the Ser-46 specific PCR primer, indicating that they possess the *Pinb-D1b* hardness allele. The majority were checked with the Gly-46 primer and none produced a product. Eight cultivars chosen at random were also checked on SDS-PAGE and were shown to express both puroindoline a and b proteins. These *Pinb-D1b* cultivars ranged in SKCS hardness from 56 ('Ute') to 84 ('Centurk'). 'Warrior', 'Scout', 'Sturdy' and Centurk (Murphy et al., 1986) were added to the original set of PNW Historical wheats. The accession of 'Triumph' was comprised of red and a minor proportion of white kernels, but both kernel color classes exhibited similar SKCS hardness (data not shown).

Two hard winter wheat cultivars, 'Chiefkan' and 'Andrews', failed to produce a product using the Ser-46 specific primers but did produce a product with the Gly-46 specific primers (Table 3). These two cultivars, then, did not possess the very prevalent *Pinb-D1b* hardness allele found in the majority (96%) of these North American hard winter wheat cultivars. Full-length puroindoline a and b PCR products were sequenced. Chiefkan proved to carry the *Pinb-D1e* allele previously observed in Canadian Red and Gehun hard spring wheat cultivars. Andrews was found to possess a single-nucleotide change (TGC to TGA) in the codon for Cys-56 which created a stop codon at this position. This Cys-56 to stop codon mutation was assigned the molecular marker designation *Pinb-D1g* (Table 1).

DISCUSSION

The molecular-genetic basis of wheat grain hardness (kernel texture) is now well established. As such, variation in puroindoline expression and DNA sequence can be used to characterize hard wheat genotypes and analyze ancestral and parental relationships. Besides the soft, wild-type sequences for puroindoline a and b (Gautier et al., 1994), four "hardness" alleles conferring hard kernel phenotype (*ha*) have been described previously (Giroux and Morris, 1997, 1998; Lillemo and Morris, 2000) (Table 1). The three most prevalent hardness mutations (*Pina-D1b*, *Pinb-D1b*, and *Pinb-D1c*) were observed among the hard spring wheats in the present study. However, no hard winter wheats with either the *Pina-D1b* or *Pinb-D1c* alleles were observed; nearly all (96%) carried the *Pinb-D1b* hardness allele. A relatively rare allele, *Pinb-D1d*, which involves a point mutation in the codon of Trp-44 such that the amino acid becomes an arginine (Lillemo and Morris, 2000) was not observed in any of the cultivars included here. Additionally, three new hardness alleles (*Pinb-D1e*, *Pinb-D1f*, and *Pinb-D1g*), all involving single-nucleotide changes, which result in stop codons in puroindoline b, were discovered in the cultivars included in the present study (Tables 1 through 3). Additional hardness mutations and molecular-genetic lesions in the puroindoline proteins beyond these will likely be discovered as broader surveys are conducted, especially among new germplasm pools and geographic regions.

In addition to those wheats considered to be of historical importance to the U.S. Pacific Northwest (Miller et al., 1990), an additional 13 spring and six winter wheats were included because of their prominent role in the development and parentage of North American wheat cultivars (Mercado et al., 1996; Murphy et al., 1986) (Tables 2 and 3). ID377s, Red Fife, and Bridger were added during the course of the study. Among the oldest cultivars included here are 'Java' (introduction 1837) and Gold Drop (introduction 1843). Clark (1927) lists Java as a hard red spring, whereas Greengenes lists it as a soft red spring. Our data are consistent with the later classification. Conversely, Clark (1927) lists Gold Drop as a soft red winter, whereas Greengenes lists it as a hard red winter; in this case our data support the former description. Certainly, cultivars even older than these which are now mostly lost to history were the very earliest soft wheats brought from Europe during the initial stages of colonization (Jones, 1946). However, since these soft wheats represent the wild-type state of the *Hardness* gene, they are of limited use (except in

an allelic sense) in studying the post-hexaploidation and domestication of wheat. If we accept the estimates that the hexaploidation event(s) were few and occurred not more than 9,000 years ago (Dvorak et al., 1998; Allaby et al., 1999), and also accept that the D-genome donor, *Aegilops tauschii* (Coss. [synonyms *A. squarrosa*, *T. tauschii*]) is uniformly soft in kernel texture, then we may conclude that all mutations in puroindoline proteins have arisen in the intervening period. Regardless of the evolutionary interest, the puroindoline hardness alleles serve to provide the means of analyzing ancestral relationships.

Among the earliest recorded introductions of spring wheats with hard kernel texture are Ladoga, Red Fife (aka Fife), Hard Red Calcutta, and Gehun (Table 2). Among these hard spring wheat cultivars, the "Fife series" is historically the most important. As related by Buller (1919), Red Fife was an inadvertent spring-habit selection from an otherwise winter-habit seed lot. The seed lot originated as a commercial sample of seed from Danzig (Gdansk) Galicia (Poland) and arrived in Ontario via Scotland about 1842. David Fife planted a portion of the seed in the spring and only one plant headed and matured. From this single plant, Red Fife originated and by the late 1880s dominated spring wheat production in Manitoba and Minnesota (Buller, 1919). Because of its popularity, Red Fife and selections thereof feature prominently in the early breeding work of Drs. William and Charles Saunders of the Dominion Experimental Farms. Further, the prevalence of the *Pinb-D1b* allele in hard spring wheats included here probably traces in large part to Red Fife and early cultivars derived from it, including Preston, Marquis, and later Thatcher. Marquis (and Thatcher) feature prominently in many North American hard spring wheat pedigrees.

Miller et al. (1990) listed White Fife and Red Fife cultivars, which were described as soft white spring and soft red spring, respectively. Consequently, neither of these cultivars were initially obtained from the PNW Historical Nursery. Mercado et al. (1996) listed Fife as a spring wheat and indicated its importance as having the largest total contribution (18.2%) to North American hard spring wheat cultivars. van Beuningen and Busch (1997) also highlighted the importance of Red Fife as an ancestor of North American spring wheat cultivars. A search of the GRIN database identified the Red Fife (PI 348919) and Fife (PI 283820) accessions included here (Table 2). Bayles and Clark (1954) described "Fife" as being another name for 'Jones Fife'; however, they describe Jones Fife as a soft red winter cultivar, as does Miller et al. (1990). It seems likely that the Red Fife and Fife included here were derived from the same original introduction and may differ little. In this regard, variants of the original "Fife" described by Fraser and Whiteside (1956) would additionally include 'Early Red Fife' ('Ottawa 16', C.A.N. 1288, a pure line selection from 'Ordinary Red Fife'), Ordinary Red Fife, White Fife (listed as a parent of 'Huron'), 'Red Fife H' (aka 'Type I c', a selection out of a common Red Fife), 'Red Fife D' (listed as a parent of Ruby), and 'White Russian' (C.A.N. 1567, aka 'Wellman's Fife', a selection out of Red Fife).

Five spring wheats were shown to carry the *Pinb-D1c* hardness allele. Ladoga was introduced by W. Saunders, Dominion Cerealist, from Russia near Lake Ladoga in 1886 (Fraser and Whiteside, 1956). Although of mixed hardness, the hard component of Ladoga was shown here to carry the *Pinb-D1c* allele. Ladoga is present in the pedigrees of Preston (Ladoga × Red Fife) and several other early cultivars developed by W. Saunders (Fraser and Whiteside, 1956). Hard Red Calcutta was also imported by W. Saunders. As Buller (1919) pointed out, "Hard Red Calcutta,... is a commercial expression and includes several different types of wheat." The sample obtained from the NSGC (CI 015090) (Table 2) carries the *Pinb-D1c* hardness allele like Ladoga. Hard Red Calcutta was the female used to cross with Red Fife in the development of Marquis. Ruby also carries the *Pinb-D1c* allele (Table 2) so its lineage is of interest. According to Fraser and Whiteside (1956), Ruby resulted from the cross 'Downy Riga' × Red Fife D made at Ottawa in 1905. Downy Riga is listed by Buller (1919) as resulting from the cross Gehun/'Onega'. Onega was introduced from northern Russia and is neither present in the NSGC nor listed on Greengenes. Gehun was found to possess a new allele and is dealt with in greater detail below. Although Downy Riga is not listed on Greengenes, a 'Riga' cultivar (CItr 3317) appears on the GRIN (received 1912 from Agriculture Canada, Brandon, Manitoba). Greengenes lists the pedigree of 'Garnet' as involving 'Preston A'/'Riga M'. From this we may conclude that the *Pinb-D1c* allele of Ruby may have come from Onega.

Red Bobs and Supreme are the other two spring wheats exhibiting the *Pinb-D1c* allele (Table 2). According to Buller (1919) Red Bobs is most likely a natural out cross between 'Bobs' and either Preston or Early Red Fife occurring at Seager Wheeler's farm in Saskatchewan. Macindoe and Brown (1968) describe Bobs as a selection from 'Blount's Lambrigg' made by William Farrer in New South Wales in 1896. Surprisingly, Blount's Lambrigg originated from a hybrid made in the 1880s by Professor A.E. Blount in Colorado. And even though Greengenes indicates that Blount's Lambrigg (aka Gypsum) was a soft white wheat, the accession included here (Table 2) possesses a significant proportion of hard kernels such that Farrer could have easily selected hard types to produce Bobs. Supreme was a plant selection from Red Bobs made by S. Wheeler (Clark et al., 1927).

Even though the *Pina-D1b* allele was fairly prevalent among the hard spring wheats studied (Table 2), the origin of this allele is less certain. 'Sea Island' is the oldest cultivar of this group. Greengenes lists it as "Probable Ladoga farmer selection." The different hardness alleles present in the accessions of Sea Island and Ladoga examined here would not support this description. The next oldest cultivar studied was 'Komar'. Greengenes lists the pedigree of Komar as Marquis/'Kota'. Since Marquis carries the *Pinb-D1b* allele from Red Fife, Kota is the logical source of the *Pina-D1b*

allele. Clark (1927) describes Kota as a hard red spring wheat, second only to Marquis in spring wheat acreage in 1924. Our data on seed obtained from NSGC (PI 192444) indicates Kota was soft. The discrepancy may lie in the identity of what really constitutes "Kota." Waldron and Clark (1919) describe the introduction of 25 samples of wheat from Russia by Prof. H.L. Bolley, one of which was approximately 75% durum and 25% common–the 25% subsequently being selected and named "Kota." The oldest accession of Kota in the NSGC is numbered CItr 5878 and described as being received from Montana St. Univ. in 1917. Analysis of this germplasm provided a SKCS hardness of 80±15 and a *Pina-D1b* puroindoline a genotype. The second accession of Kota in the NSGC, PI 192444 and the one used here, is listed as coming from Portugal in 1950. The third, PI 341423, is described as being collected from Turkey and was received in 1969. Clearly, in the context of ancestral lineages, CItr 5878 is the correct cultivar and likely represents the parent of Komar. Kota is then the oldest confirmed direct introduction of the *Pina-D1b* allele. 'Ceres' having the same pedigree (Marquis/Kota) (Greengenes), carries the *Pinb-D1b* allele.

The last two *Pina-D1b* cultivars which were introduced to North America are Red Egyptian and Marroqui 588. Both are listed by Mercado et al. (1996) as featuring prominently in North American hard spring wheats but make no mention of the specific contribution. A cursory search of Greengenes revealed that Red Egyptian appears in the pedigree of RL 4205 which in turn appears in the pedigree of 'Grandin', 'Alex', 'Butte 86', and 'Len'. Although none of these cultivars were included here, Giroux and Morris (1998) showed that Butte 86 carried the *Pina-D1b* allele. The GRIN lists five accessions of "Red Egyptian." The oldest, CItr 12345 which was included here, came to the NSGC via Australia in 1944. Three (PI 45374, PI 45403, and PI 45415) came from South Africa in 1917. The fifth, PI 192020, came from Ethiopia via Italy in 1950. Our ability to establish ancestral lineage among these accessions and existing cultivars is poor.

Marroqui 588 (aka 'Marroqui 588 Selection') is described by GRIN as being collected in Mexico in 1948 by the USDA. A search of GRIN for accessions with "Marroqui*" in their pedigree yielded 179 accessions, mostly numbered lines from CIMMYT. Of note, 'Yaqui 50' has the pedigree 'Newthatch' / Marroqui 588.

In our survey of spring wheat cultivars, two new hardness alleles were discovered. Gehun and Canadian Red shared a common point mutation in the codon of Trp-39 which created a stop codon. Fraser and Whiteside (1956) and Buller (1919) indicate that Gehun was introduced from India by W. Saunders and used in the cross that produced Prelude ('(downy) Gehun'/'Fraser', cross made in 1903) and in one of the parents of Ruby (Downy Riga/Red Fife D, cross made in 1905; Downy Riga = Gehun/Onega). The GRIN lists four Gehun accessions. The first three were collected in India, but from 1936 to 1948. The fourth accession was obtained from the Australian Winter Cereals Collection. The connection between these accessions and the Gehun used by Saunders is uncertain, but probably has no direct connection. Greengenes and the GRIN list Canadian Red as a hard white spring wheat from F.G. Stokes, Kelseyville, CA, of unknown pedigree, and released in 1919.

The last spring wheat of notable interest is Utac. The samples of Utac from both the PNW Historical Nursery and the NSGC were both shown to be mixed in kernel texture. The hard component was isolated and sequencing of puroindoline genes revealed that it possessed a new hardness allele, designated *Pinb-D1f* (Table 1). Similar to the *Pinb-D1e* allele, the *Pinb-D1f* allele is also a point mutation which creates a stop codon, in this case at position 44 which is only four bases away from the first hardness mutation characterized in puroindoline b, *Pinb-D1b* at position 46 (Giroux and Morris, 1997) (Table 1). This mutation occurs in the same codon as the apparently rare *Pinb-D1d* allele (Trp-44 to Arg-44, Table 1) (Lillemo and Morris, 2000). Bayles and Clark (1954) describe Utac as a club cultivar with, "kernels white, midlong, semihard to hard," which resulted from a cross between 'Dicklow' and 'Sevier' made about 1923 at the Utah Agricultural Experiment Station, Logan, UT. It was released in 1928. Dicklow is described by Greengenes and Bayles and Clark (1954) as a soft white spring wheat. Sevier (CItr 6247) was obtained from the NSGC, the puroindolines sequenced, and was found to carry the same *Pinb-D1f* allele. The origin of Sevier, a Utah landrace dating to about 1888, is provided by Stewart (1923).

As noted from Table 3, the hard winter wheats of North America mostly owe their hard kernel texture to Turkey, Kharkof, and other similar wheats referred to as the "Crimean Group" which were first introduced by immigrants to Kansas in 1873 (Bayles and Clark, 1954). Yet, surprisingly, the current accessions of Turkey and Kharkof are clearly mixtures of hard and soft alleles (Table 3). Whether these cultivars existed as such when first introduced or whether they became mixed through subsequent propagation is a matter of conjecture. Certainly as early as 1915, 'Montana No. 36' and other such direct selections from Turkey were uniformly hard-kernel cultivars (as further example, Cheyenne was a single plant selection from 'Crimean' made in 1922). As an aside, we can predict that the *Pinb-D1b* allele found in the mapping population involving 'Clark's Cream' derived from Turkey wheat (Campbell et al., 1999).

The only hard winter wheat cultivars not carrying the *Pinb-D1b* allele were Chiefkan and Andrews (Table 3). Sequencing puroindoline a and b revealed that Chiefkan carries the same *Pinb-D1e* hardness allele as Canadian Red and Gehun (Table 1). Bayles and Clark (1954) list the pedigree of Chiefkan as 'Blackhull'/soft wheat// 'Superhard Blackhull'. 'Kanhull', a sister selection to Chiefkan (Bayles and Clark, 1954), was not included in this study.

Andrews was the other hard winter wheat that did not possess the prevalent *Pinb-D1b* allele. Again, the puroindoline genes were sequenced and once more a point mutation which caused a stop codon in puroindo-

line b was found; in this case, at position Cys-56. The pedigree given for Andrews is PI 167822/CI 13438/2/CI 19342/'Itana'/3/CI 17271/Sturdy. 'Itana 65' (and presumably, Itana) and Sturdy carry the *Pinb-D1b* allele (Table 3).

It will be of particular interest to those engaged in genetically improving wheat to learn to what extent the various puroindoline hardness alleles confer superior end-use quality. In this regard, the results of Giroux et al. (2000) and Martin et al. (2000) indicate that the *Pinb-D1b* allele confers significantly softer grain (Near-Infrared Reflectance and SKCS), higher milling break flour yields, and higher flour yields compared with the *Pina-D1b* allele. The production of near-isogenic lines involving all hardness alleles in a common genetic background would help advance this line of research.

In summary, the prevalence of puroindoline hardness alleles among North American wheat cultivars provides insight as to their ancestral lineage, a means of characterizing more fully their genome and highlights the relative greater diversity of hard spring wheat introductions as opposed to the hard winter wheats of the Great Plains, where Turkey, its descendants and closely related types account for the hard kernel texture of all but two cultivars examined. In this survey of wheat cultivars of historical importance, six different hardness alleles were encountered, three being previously unreported.

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Milling and Bread Baking Traits Associated with Puroindoline Sequence Type in Hard Red Spring Wheat

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ABSTRACT

Recent results have shown that mutations in genes coding for puroindoline a and b (*PinA* and *PinB*) are associated with the expression of the hard texture of wheat (*Triticum aestivum* L.) grain. A majority of hard wheats have a glycine-to-serine mutation in puroindoline b (allele *PinB-D1b*), or they are devoid of puroindoline a (allele *PinA-D1b*). Hard wheats with *PinA-D1b* tend to be harder than those with *PinB-D1b*. Grain hardness is known to affect milling and baking traits. Our objective was to determine the influence of allelic variation in *PinA* and *PinB* on milling and bread quality traits in a recombinant inbred population segregating for *PinA-D1b* and *PinB-D1b*. One hundred thirty-nine recombinant inbred lines from the cross 'Butte 86' (*PinA-D1b* allele)/ND2603 (*PinB-D1b* allele) and parents were grown in a field trial with two replications at two locations. Grain hardness was measured by near-infrared reflectance (NIR) and the single-kernel characterization system (SKCS). Grain was milled and baked for each line. Puroindoline allele type was determined for each line. The *PinB-D1b* group had significantly softer grain, higher break flour yield, flour yield, milling score, and loaf volume, and lower flour ash and crumb grain score (low score being desirable) than the *PinA-D1b* group. Significant genetic variability was detected within allelic classes for all traits. The proportion of variation among entry means attributed to puroindoline classes was 34% for break flour yield, 26% for NIR hardness, and 22% for SKCS harness index. Grain hardness was negatively correlated with break flour yield, flour yield, and mixing score and positively correlated with flour ash. Grain hardness was not correlated with loaf volume or crumb grain score. The *PinB-D1b* allele was more desirable for milling and bread baking, although superior milling and bread quality genotypes could be selected within either class.

Wheat is classified into hard and soft classes on the basis of the texture of the grain. These textural classes coincide with differences in milling and end-use properties (reviewed in Pomeranz and Williams, 1990; Morris and Rose, 1996). The distinction between soft and hard classes of wheat is governed by the Hardness (*Ha*) locus on chromosome 5DS (Mattern et al., 1973; Law et al., 1978) with additional modifying genes contributing to variation within classes (Symes, 1965; Baker, 1977); however, Baker and Sutherland (1991) and Giroux et al. (2000) observed significant genetic variation for grain hardness within crosses of hard wheats.

Greenwell and Schofield (1986) identified friabilin as a marker protein for grain softness which was present in larger amounts on the surface of water-washed starch of soft wheats than from hard wheats (Bettge et al., 1995; Greenblatt et al., 1995; Morris et al., 1994). Friabilin is composed of two major polypeptides termed puroindoline a and puroindoline b. Genes coding for these two proteins, *PinA* and *PinB*, are tightly linked to the *Ha* locus on chromosome 5D (Jolly et al., 1993; Sourdille et al., 1996) and probably function together as the *Ha* locus. Recent results have shown that mutations in *PinA* and *PinB* are associated with the expression of hard texture. Giroux and Morris (1997, 1998) showed that hard texture was completely linked to a glycine-to-serine mutation in puroindoline b (allele *PinB-D1b*), or the complete absence of the puroindoline a protein (allele *PinA-D1b*). In a survey of hard wheats, cultivars with the *PinA-D1b* allele were on average 7 units harder than those with *PinB-D1b* (Giroux and Morris, 1997; unpublished results). Giroux et al. (2000) further showed that progeny carrying the *PinA-D1b* allele averaged 4.5 units harder than progeny with *PinB-D1b* in three hard red spring crosses segregating for *PinA-D1b* vs *PinB-D1b*. A more recent survey has found additional mutant alleles of *PinA* or *PinB* linked to hard textured grain (Lillemo and Morris, 2000).

Since kernel texture has been shown to be associated with numerous milling and bread quality traits in hard wheats (Slaughter et al., 1992) and Giroux et al. (2000) showed hard wheats with the *PinA-D1b* allele tend to be harder than those with the *PinB-D1b* allele, it is possible that allelic variation at the *PinA* and *PinB* loci

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Abbreviations: NIR, Near-infrared reflectance; SKCS, Single Kernel Characterization System.